Full Paper

Anti-arthritic Effects of *Ephedra sinica* STAPF Herb-Acupuncture: Inhibition of Lipopolysaccharide-Induced Inflammation and Adjuvant-Induced Polyarthritis

Mi-Jung Yeom¹, Han-Chang Lee¹, Gun-Ho Kim¹, Hye-Jung Lee¹, Insop Shim², Seung-Kyu Oh¹, Sung-Keel Kang³, and Dae-Hyun Hahm^{1,*}

¹Laboratory of Acupuncture & Meridian, Department of Oriental Medical Science, Graduate School of East-West Medical Science, Kyung-Hee University, Kihung-up, Yongin-si, Kyungki-do 449-701, Korea

²Department of Integrative Medicine, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea

³Department of Acupuncture & Moxibustion, College of Oriental Medicine, Kyung-Hee University, Seoul 130-701, Korea

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Abstract. Anti-inflammatory and anti-arthritic effects of water distillates of *Ephedra sinica* STAPF (ES), in herb-acupuncture, on the inflammatory responses of arthritis was investigated using phorbol 12-myristate 13-acetate (PMA)/lipopolysaccharide (LPS)-induced human macrophage and adjuvant-induced arthritic rat. The luciferase reporter vectors driven by the tumor necrosis factor (TNF)- α and cyclooxygenase-2 promoters were transiently transfected into U937 cells, which were then differentiated and stimulated by PMA and LPS, respectively, to develop an in vitro anti-inflammation assay system. The luciferase activities, observed in the activated U937 cells, were significantly inhibited by ES herb-acupuncture, compared to those of PD98509 and berberine. To evaluate ES herb-acupuncture as a novel anti-arthritic therapy, a polyarthritic rat model was developed using heat-killed *Mycobacterium tuberculosis*, and 50 μ l of ES distillate was subcutaneously injected into the ST36 acupoint on each knee joint. While the articular indexes of arthritic rats were evidently decreased by ES herb-acupuncture, their body weights did not regain their initial levels. This may be due to the accelerating effects of ES on weight-loss and fat consumption. The mRNA expressions of TNF- α and interleukin (IL)-6 genes, which were closely stimulated in the arthritic rat joints, were found to be restored to the normal levels through the ES treatment. In the case of IL-1 β , the recovery was not significant but substantial. The anti-arthritic effect of ES herb-acupuncture was not found in the ES-treated/non-acupoint group. In conclusion, the ES herb-acupuncture into the ST36 acupoint was found to be effective in alleviating the inflammatory response and thus arthritic symptoms in adjuvant-induced arthritic rats.

Keywords: *Ephedra sinica* STAPF, herb-acupuncture, adjuvant-induced rat arthritis, inflammation, cytokine

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease. In human as well as in animal

models, RA is characterized by a series of pathological processes of the joints, such as leukocyte infiltration, a chronic inflammation, pannus formation, and extensive destruction of the articular cartilage and bone. Although the exact cause of RA has not been elucidated in detail, pro- and anti-inflammatory cytokines seem to play an important role in the etiology of the disease. In particular, it was reported that the inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-

*Corresponding author. Present address for correspondence: Neuroscience Research Institute, Gachon Medical School, 1198 Kuwol-dong, Incheon 405-760, Korea. dhahm@paran.com

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1 β , and IL-6, play key roles in the inflammation and joint damages during the development of RA (1–6).

The most commonly prescribed medication for RA treatment is non-steroidal anti-inflammatory drugs (NSAIDs) (7). While these drugs effectively reduce the symptoms such as joint pain, swelling, tenderness, and morning stiffness, they may cause serious side effects such as gastrointestinal ulcer and renal morbidity (8, 9). Accordingly, reducing side effects should be considered while designing improved therapeutics for RA, besides enhancing medicinal effectiveness.

In the traditional medicine of many Asian countries, acupuncture is considered as one of the most effective medicinal treatments, especially for chronic diseases accompanied with severe pain, for example, sprain, osteoarthritis, and rheumatoid arthritis (10, 11). Electrical or manual stimulation of an acupuncture point (acupoint) is the most popular form of acupuncture therapy (12). Recently, a certain group of physicians in oriental medicine reported that chemical stimulation of acupoints by subcutaneous injection of distilled herbal extracts, referred to as herb-acupuncture or aqua-acupuncture in Korea, produces dramatic and synergistic effects that treat many degenerative and chronic diseases (8, 12, 13).

The herbal extracts, used in the herb-acupuncture, are manufactured by repeated water distillation to obtain extracts of extreme purity, and only tiny amounts of the herbal extracts are injected into a specific point (acupoint) of the body (14). Accordingly, both medicinal benefits of acupuncture and herbal therapy can be attempted by a single treatment of herb-acupuncture. However there have been little systematic and scientific studies on the therapeutic effectiveness of herb-acupuncture, as of yet.

Ephedra sinica STAPF (ES), also known as *Ma-huang*, is famous for containing many alkaloids of the ephedrine series (15). ES has traditionally been used in oriental medicine for thousands of years, for example, to induce perspiration and as an anti-allergy agent. These days, it is a major ingredient of herbal formulas used to relieve pain and headaches, to control body weight, to lower fever, and to alleviate inflammatory responses (16, 17). Although there have been several reports about the anti-inflammatory activities of ES, little is known about the medicinal effect of ES on the inflammatory diseases, including arthritis, at molecular levels.

The ST36 acupoint (*Zusanli*), selected for ES herb-acupuncture in this study, is one of the most effective acupoints with a wide range of medicinal effects, for example, spasmolytic and analgesic effects on the gastrointestinal tract, and homeostatic effect in the endocrine system and metabolism. It has been also used

for the treatments of aching of the knee joints and legs in Korean traditional medicine (8, 10, 11, 18, 19).

In the present study, anti-arthritic effects of ES herb-acupuncture were investigated by using an in vitro phorbol 12-myristate 13-acetate (PMA)/lipopolysaccharide (LPS)-induced human macrophage cell line including a luciferase reporter vector that was transcriptionally fused to TNF- α or cyclooxygenase (COX)-2 promoter and an in vivo adjuvant-induced arthritic (AIA) rat model. The inhibitory activity of ES distilled extracts on the TNF- α and COX-2 gene expressions was analyzed by measuring the luminescence in vitro and then verified in rats by confirming the medicinal effect of ES herb-acupuncture, that is, the injection of ES extracts into an acupoint, compared to the treatment to a non-acupoint.

Materials and Methods

Animals and reagents used

Female Sprague-Dawley rats (Samtaco Co., Osan, Korea) weighing 130–150 g were used for the experiments. Animals were given a period of 1 week to adjust to the new environment ($22 \pm 2^\circ\text{C}$, 45%–60% humidity, and 12-h light / 12-h darkness cycle) and provided with food and water ad libitum. The experimental procedures were carried out according to the animal care guidelines of the NIH and the Kyung Hee University Institutional Animal Care and Use Committee. PMA, LPS (from *Escheichia coli*), PD98059, and berberine chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). The dried herb of ES was kindly supplied from Kyung-Hee University Oriental Hospital (Seoul, Korea), and voucher specimens have been deposited in the Herbarium of Kyung-Hee University. The distilled water extracts of ES for herb-acupuncture treatment were manufactured in an ampoule by the Korean Institute of Herbal Acupuncture (Seoul, Korea). The manufacturing processes were sequentially boiling, evaporation, condensation, and sterilization steps, which were always under the quality control of assuring that there were no suspended particles detectable by visible spectrophotometer. The extraction was started with 200 g of dried ES and finally produced 300 ml of ES distillates for herb-acupuncture. By the regulations of the Korean Institute of Herbal Acupuncture, the ES distillates passed the germ-free, anti-histamine, and endotoxin-free tests; and thus they were accepted as an s.c. injection grade for humans.

Cell culture and transient transfection

U937 cells were obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 (Gibco

BRL, Grand Island, NY, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone Laboratories Co., Logan, UT, USA), penicillin (100 units/ml), and streptomycin (100 units/ml). The cells were split at 70% – 80% of confluency. For transfection, U937 cells (5×10^6 per well) were co-transfected with 2 μ g of the luciferase reporter vector and 1 μ g of pSV β -gal vector as a control using EffecteneTM (Qiagen, Valencia, CA, USA).

Plasmid construction

The TNF- α and COX-2 promoters (nucleotides -1364 to -36 and -7417 to -45, respectively) were generated by PCR using human chromosomal DNA as a template and 5'- and 3'-primers, designed to contain the restriction enzyme sites of *Kpn* I and *Hind* III at both ends. Each of the PCR products was subcloned into the pGL3-basic vector (Promega Co., Madison, WI, USA) (Fig. 1).

Luciferase assay

Twenty-four hours after transfection of U-937 cells with vector DNA, the cells were washed with phosphate-buffered saline (JBI Co., Daegu, Korea), and then equal aliquots of the cells were placed in the individual wells of a 12-well plate, each containing 25 nM PMA. After 24 h, each well was treated with ES extracts, PD98059, or berberine. After 2-h stimulation of the cells in each plate with LPS (5 μ g/ml), the cells were harvested,

and lysed for analyzing luciferase activity. Luciferase activity was measured by using a commercialized assay kit, Luciferase Reporter Assay SystemTM (Promega Co.) and normalized by using β -galactosidase activities of the same lysates.

Induction of adjuvant-induced polyarthritis in rat

The adjuvant-induced polyarthritis was induced as previously described (13). Rats were intradermally injected at the base of the tail with 1.5 mg of heat-killed *Mycobacterium tuberculosis* H37 RA (Becton Dickinson, Sparks, MD, USA), emulsified in 0.15 ml squalane (Sigma-Aldrich Co., St. Louis, MO, USA). Control rats were similarly treated with the vehicle only. The first day of arthritis induction was considered as day 0. On day 12, the animals exhibiting the similar severity of arthritic symptoms were selected for further experiments, so that the rats' knees in each arthritic group exhibited a similar pathological condition of arthritis. All time points were considered relative to the induction day of adjuvant-induced arthritis, which were designated as day 0.

Experimental groups and ES-acupuncture treatments

Experimental animals were divided into four groups: 1) saline-treated non-arthritic animals (Cont, n = 6); 2) saline-treated arthritic animals (AIA, n = 6); 3) ES-treated/ST36 acupoint (*Zusanli*) arthritic animals

Fig. 1. Construction of luciferase reporter vector including TNF- α or COX-2 promoter using pGL3-basic vector.

(ES/Z, $n = 6$); and 4) ES-treated/non-acupoint arthritic animals (ES/B, $n = 6$). At the onset stage of arthritis, when the redness and swelling of the joints and the body weight loss usually start to appear, the adjuvant-induced arthritis rats were randomly divided into three groups such as AIA, ES/Z, and ES/B. Then, the ES treatment was performed in the ES/Z and ES/B groups to examine the therapeutic effect of ES injection into the ST36 acupoint (20). The ES treatment was initiated on day 12 post adjuvant injection (onset stage of arthritis) and applied to both legs once daily for 15 consecutive days. The ST36 acupoint is located near the knee joint of the hind limb, 2-mm lateral to the anterior tubercle of the tibia (11). The 50 μ l of the distilled extract of ES was subcutaneously injected into an ST36 acupoint on each knee joint of the leg. Rats in the CONT and AIA groups were treated with saline into the same acupoint and rats in the ES/B group were subcutaneously treated into an arbitrary non-acupoint, located near the midline on the back. A 0.5-ml insulin syringe with 30 gauge 5/16" (8 mm) needle (BD Medical Systems, Franklin Lakes, NJ, USA) was used for ES or saline injections.

Arthritis evaluation

The clinical symptoms of arthritis in all four limbs were evaluated according to visual appearance by two observers, who were unaware of the identity of the animal groups. The scores were rated on a scale of 0–4 for each knee joint: 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, and 4 = excess edema with joint rigidity (21). The mean articular index score was given for each foot of adjuvant-induced arthritis rat. The total score for each animal was then calculated and used as an articular index with a maximum value of 16. Body weights were regularly measured by a digital balance after the adjuvant injection. Body weight and articular index were assessed at the intervals of one or two days.

RT-PCR analysis

The rats were killed on day 28 after the adjuvant injection. The paws of the rats were harvested by using a surgical scissor. After removals of skin and muscle tissue, the knee joints were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from knee joints of rats using TRIzol™

Table 1. Primer sequences, operating conditions, and PCR product sizes

Gene name	Oligonucleotide sequences (5'–3')	Denaturing temperature and time ($^{\circ}\text{C}$, s)	Annealing temperature and time ($^{\circ}\text{C}$, s)	Elongation temperature and time ($^{\circ}\text{C}$, s)	Cycle number	Product size (base pair)	GenBank number
hGAPDH	F-ATCCCATCACCATCTTCCAG R-CCTGCTTACCACCTTCTTG	94 (30)	50 (30)	72 (45)	30	579	NM_002046
hTNF- α promoter	F-CACGGAGGTACCTGCACCTCGATGAAGC R-GTGTGCCAAAAGCTCTTATATGTCCCTGGG	94 (60)	60 (45)	72 (60)	30	1,328	GI:339739
hCOX-2 promoter	F-GGCAGATGGTACCTTCTAGAAATTCATC R-CCGAATAGAGAAGCTTCGTGACTTGG	94 (60)	60 (45)	72 (420)	30	7,372	GI:4468336
hTNF- α	F-CCTCAACCTCTTCTGGCTCA R-CTGGGCTCCGTGTCTCAA	94 (30)	57 (30)	72 (45)	30	344	BC 028148
hCOX-2	F-CCAAGTCACGAAGCTTCTCTATTCGG R-GGCAGATGGTACCTTCTAGAAATTCATC	94 (30)	58 (30)	72 (45)	35	341	NM_007590
mGAPDH	F-ATGGGAAGCTGGTCATCAAC R-GGATGCAGGGATGATGTCT	94 (30)	58 (30)	72 (60)	30	440	NM_017008
mTNF- α	F-GTCGTAACCAACCAAGC R-GACTCCAAAGTAGACCTGCCC	94 (45)	58 (45)	72 (90)	35	393	AY 427675
mIL-1 β	F-GGCATAACAGGCTCATCTGG R-CATCATCCACGAGTCACAG	94 (45)	58 (45)	72 (90)	35	414	NM_031512
mIL-6	F-CCACTGCCTTCCCTACTTCA R-TGGTCCTTAGCCACTCCTTC	94 (60)	63 (60)	72 (120)	37	498	NM_012589

hGAPDH: human glyceraldehyde-3-phosphate dehydrogenase; mTNF- α : murine tumor necrosis factor α ; COX-2: cyclooxygenase 2; IL-1 β : interleukin-1 β ; IL-6: interleukin-6.

(Invitrogen Co., Carlsbad, CA, USA) following the manufacturer's protocol and then treated with DNase (RNase-free; Takara Co., Shiga) to remove the contaminating genomic DNA. One microgram of total RNA from rat knee joint was reverse-transcribed into cDNA using 200 units of M-MLV reverse transcriptase (Invitrogen Co.) in 20 μ l of reaction volume. The cDNA was amplified in 20 μ l of reaction mixture including 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.4 μ M each of the 3' and 5' primer, and 0.5 U Taq polymerase (Takara Co.) using a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA, USA). All primers were designed using the published cDNA sequences and the primer selection software, offered through a web site, Primer 3 (<http://www.genome.wi.mit.edu>, The Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The primer sequences and operating conditions are listed in Table 1. PCR products were separated on 1% agarose gels, stained with ethidium bromide, and photographed. The intensities of bands were measured by using ImageMaster VDS™ (Amersham Biosciences, Uppsala, Sweden) with an image analysis software, ImageMaster TotalLab™ (Amersham Biosciences). The amounts of target PCR products were normalized against GAPDH PCR product in the corresponding samples.

Data analyses

The data were presented as the mean $\pm$ S.E.M. The significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc analysis. Difference was considered significant at $P < 0.05$.

Results

In vitro inhibition activity of ES herb-acupuncture on PMA/LPS-induced inflammation

It was well known that TNF- α and COX-2 genes are differentially expressed in response to various inflammatory stimuli including LPS. The U937 cell is a macrophage branch of myeloid lineage that can be induced to secrete inflammatory cytokines by exposure to a variety of mitogenic agents including PMA, bacterial LPS, and okadaic acid. In addition to the role of being a strong induction signal for cytokine transcription, PMA can be used as an exogenous signal to induce myelocytic cells to be differentiated from a promonocytic to a monocytic stage of development by a mechanism dependent on the activation of protein kinase C (PKC) (22, 23). According to the previous studies, TNF- α and COX-2 mRNA expressions are not stimulated by LPS

unless the cells are differentiated by PMA in U937 cells (24, 25). Therefore the inflammatory condition of U937 cells was developed by the sequential treatment of PMA and LPS. In order to verify the intrinsic response to PMA/LPS treatment in U937 cells, the gene expressions of TNF- α and COX-2, stimulated by PMA/LPS, were preliminarily confirmed by RT-PCR analysis of mRNAs of those genes in the differentiated U937 cells (data not shown). The *in vitro* screening system for anti-inflammatory and analgesic activities of herbal distillates for herb-acupuncture was designed using a transient reporter gene expression system driven by TNF- α and COX-2 promoters, respectively. The luciferase gene in pGL3-Basic was transcriptionally spliced to the downstream of TNF- α and COX-2 promoters in frame as shown in Fig. 1, and then the vectors constructed were transiently transfected into the U937 cell line. The highly purified distillates of ES water extracts for herb-acupuncture, which have passed tests to demonstrate that they are germ-free, anti-histamine, and endotoxin-free, and thus certified for human use as an s.c. injection, were supplied by Korean Institute of Herb-Acupuncture. The berberine chloride (transcriptional COX-2 inhibitor) and PD98059 (transcriptional TNF- α inhibitor) were used as positive controls (26, 27). As shown in Fig. 2, the TNF- α and COX-2 driven luciferase transcriptions were significantly inhibited by the *in vitro* treatment of ES herb-acupuncture in PMA/LPS-induced U937 cells, as compared to PD98059 and berberine chloride, respectively. In the

Fig. 2. Inhibitory effects of *Ephedra sinica* (ES) distilled extracts, in herb-acupuncture, on TNF- α (A) and COX-2 (B) expression in PMA/LPS-induced inflammation. ES1 is ES-distillate without dilution. ES 0.1 and ES 0.01 mean 1/10 and 1/100 diluted ES-distillates, respectively. Fifty micromolar berberine or PD98051 was used as a positive control. Values are means with standard deviations from at least three independent experiments.

case of COX-2, ES-herb acupuncture exhibited an even higher inhibition activity than berberine, an alkaloid present in a number of clinically-important medicinal plants, including *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). Little inhibition activities were observed in the treatments of 1/10 and 1/100 diluted solutions of ES distillates (Fig. 2).

Evaluations of ES herb-acupuncture in AIA rat

In order to evaluate the therapeutic effects of ES herb-acupuncture on the progression of arthritic disease in AIA rats, two different pre-clinical parameters, such as body weight and articular index, were monitored as apparent indicators of arthritic symptoms. It was previously reported that the redness and swelling of

the arthritic joint and the loss of body weight usually began to appear at the onset stage of arthritis (19). In this study, the arthritic rat joints exhibited the typical swelling patterns of rat polyarthritis, which started to increase on day 12 and reached a maximum plateau on day 21, after the adjuvant injection (day 0) (Fig. 3). The ES herb-acupuncture treatment was initiated at the onset stage of polyarthritis development (day 12). The ES-treated/ST36 acupoint arthritic rats (ES/Z group) did not regain their original body weights, even though their apparent symptoms improved significantly. The ES-injected groups (ES/Z and ES/B), irrespective of injection point used (ST36 acupoint or non-acupoint), exhibited the same progression patterns of body weights and they exhibited low recovery of their body weights after ES acupuncturing, compared to the saline-treated arthritic group (AIA). This may be due to the accelerat-

Fig. 3. Effect of *Ephedra sinica* (ES) on articular indexes of AIA rats. ES treatment was started day 12 post adjuvant injection and injected once in a day for 17 days. ES (0.1 ml) was administered into either the *Zusanli* acupoint (ES/Z) or an arbitrary non-acupoint on the back (ES/B). Data were presented as the mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA. * P <0.05 and ** P <0.01, as compared to the AIA group.

Fig. 4. Effect of *Ephedra sinica* (ES) on body weights of AIA rats. ES treatment was started day 12 post adjuvant injection and injected once in a day for 17 days. ES (0.1 ml) was administered into either the *Zusanli* acupoint (ES/Z) or an arbitrary non-acupoint on the back (ES/B). Data were presented as the mean $\pm$ S.E.M.

ing effects of ES on weight-loss and fat consumption. To precisely assess the pathogenic condition of arthritic disease, the articular index scores were determined regularly throughout the whole period of arthritis development because the articular index is one of the accurate indexes to evaluate arthritic symptoms. The articular index of the AIA group rapidly increased from day 10 to day 21, which means that the polyarthritic symptoms certainly developed (Fig. 4). During the initial phase of ES herb-acupuncture treatment (day 12 – 16), the articular indexes of the ES/Z group showed little difference with those of the AIA group. However, after this phase, the indexes started to decrease and finally reached to 50% of those of the AIA group.

Inhibitory effect of ES herb-acupuncture on TNF- α , IL-1 β , and IL-6 mRNA expressions

Anti-inflammation activity of ES herb-acupuncture was investigated by RT-PCR analysis of several cytokines at the transcription level. In a pathogenic cascade of rheumatoid arthritis, the inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, are secreted from synovial tissues for signaling the release of tissue-damaging enzymes responsible for cartilage destruction. Thus, the controls of inflammatory cytokine expressions in arthritic joint are considered to be one of the important target points in designing new therapeutic medicines for rheumatoid arthritis as well as to assess the arthritic symptoms with a molecular aspect. On day 28, after adjuvant injection, the mRNA expression levels of the cytokines were analyzed by RT-PCR using the total RNA samples prepared from the whole part of the knee joint of hind paw, including synovial membrane, synovial fluids, and cartilage. As shown in Fig. 5, the

gene expressions of TNF- α , IL-1 β , and IL-6 were dramatically stimulated in the AIA group as the arthritis progressed. Whereas the non-acupoint stimulation by ES herb-acupuncture (ES/B) did not show any significant decrease of the cytokine mRNA expression, as compared to the AIA group, the ST36 acupoint stimulation (ES/Z) produced significant repression of the cytokine mRNA expression. It was precisely coincident with the experimental results of articular index measurement. In the case of TNF- α , the mRNA expression levels in the ES/Z group were almost the same as those in the CONT group, which meant the ST36 acupoint stimulation completely suppressed the induction of TNF- α gene due to adjuvant injection. There were not any significant differences in these cytokine expression profiles between fore and hind foot (data not shown).

Discussion

Since AIA of rat is a typical animal model, widely used for studies of rheumatoid arthritis, autoimmune diseases, and inflammation, this model has been used to examine and evaluate newly developed anti-arthritic drugs (21, 28). In the present study, the anti-arthritic activities of ES herb-acupuncture, a novel type of acupuncture, were investigated using an AIA rat model. In the basic principles of herb-acupuncture, both medicinal effects of acupuncture and herbal therapy were synergically attempted by a single treatment of herb-acupuncture. The ES herb-acupuncture on the specific acupoint (ST36) was thus expected to exert therapeutic efficacy to dramatically treat chronic diseases including rheumatoid arthritis.

The principal function of the herbal component in

Fig. 5. Expressions of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) mRNAs in hind paws of adjuvant-induced arthritic rat on day 28. Control: lane 1, AIA: lane 2, ES/Z: lane 3, ES/B: lane 4. Each PCR product was normalized to GAPDH. The observed bands were verified on the gels, with the predicted sizes of GAPDH (579 bp), TNF- α (414 bp), IL-1 β (393 bp), and IL-6 (498 bp). Values are means with standard deviations from at least three independent experiments. Statistical analysis was performed by one-way ANOVA. *** P <0.001 vs AIA group, and # P <0.01 and ### P <0.001 vs control (normal) group.

herb-acupuncture was the effective and prolonged stimulation of acupoints on the skin, as well as its intrinsic pharmacological effects. This might be one of the reasons why the evaporated fraction of herbs, boiled in water, was utilized in herb-acupuncture. As for the chemical components of ES herb-acupuncture, HPLC analysis was tried to identify the major component of ES distillates for anti-inflammatory or anti-arthritic activity. However, under the operating condition of HPLC, that is, MD-150 column (ESA, Inc., Chelmsford, MA, USA), 100 μ l of injection volume, 0.7 ml/min of acetonitrile/water gradient, and UV & EC detectors, only one small peak, that was almost indistinguishable from the noise peaks, with very early retention time was detected (data not shown). This meant that due to the intrinsic property of herb-acupuncture, extremely low concentration of each solute existed in the evaporated part of ES herb-acupuncture solution, which could not be separated or even detected by conventional methods.

To investigate the pharmacological properties of the evaporated fraction, which might have quite a few and a little volatile components of ES, an in vitro assay system assessing the inflammatory response was developed using the human monocyte cell line U937 and luciferase reporter vector. The anti-inflammatory effect was significantly observed only in the original solution of ES distillate without dilution, implying that the distilled fraction was prepared in a highly diluted state. In the case of inhibiting the TNF- α expression, the original solution of ES distillate showed almost the same degree of the inhibition as PD98051, a strong inhibitor of TNF- α transcription.

As an herbal component of herb-acupuncture, *Ephedra sinica* (ES), also known as *Ma-huang*, was used in this study. It is a botanical resource of ephedrine alkaloids, used as a natural stimulant or a thermogenic diet aid (29). The primary use of its products has been for weight loss, weight management, and energy enhancement for thousands of years in traditional oriental medicine (16, 17). However, the adverse effects, which are related to the consumption of ephedra as a dietary supplement, have been reported ranging from minor complaints such as palpitation to death. The adverse effects appear to be the result of overuse, inconsistent potency, individual sensitivity, interactions with drugs and other products, and contaminations included (16).

Thus ES herb-acupuncture therapy could be a kind of alternative ES therapy to minimize the adverse effects of ES as well as to evaluate the medicinal effect of ES as an alleviative agent for arthritis. Herb-acupuncture is a new acupuncture therapy to stimulate the acupoints with minute amount of herbal extracts in Korean and Chinese

traditional medicine. It is thus thought to attempt both effects of acupuncture and herbal treatments in one try (13).

In this study, the single acupoint of *Zusanli* (ST36) was used to evaluate the therapeutic effectiveness of the ES herb-acupuncture. The *Zusanli* acupoint was selected on the basis of many scientific studies about its anti-arthritic or analgesic effects (8, 13, 18). In traditional Chinese and Korean medicines, *Zusanli* is one of the most frequently used acupoints and certainly the most intensively studied single acupoint in acupuncture and moxibustion therapies (11). Moreover, this acupoint has the advantage of being relatively easy to find in small animals, including rats and mice (13).

It was previously reported that the analgesic effect of acupuncture on chronic pains is mediated by neural transmission, mostly involving the opioid system (30). Even though this mechanism can explain the parts of the medicinal effects of acupuncture, which are usually spread to a long distance in the body, this mechanism does not cover many observations of anti-nociceptive therapy by acupuncture, for example, the acupuncture efficacy by ipsilateral stimulation of acupoints and the significant improvement of analgesic effect by various acupuncture manipulations such as rotation, varying depth, and duration or angle of the insertion. Recently, it was reported that the stimulation of the vagus nerve could inhibit significantly and rapidly the release of macrophage TNF, and thus attenuated inflammatory responses through the acetylcholine-mediated (cholinergic) receptor (31). It implies that if the acupuncture or electroacupuncture stimulates the vagus nerve in peripherals, it can control the diverse human diseases accompanying the excessive inflammation response, including rheumatoid arthritis.

In our study, the ES herb-acupuncture showed the anti-arthritic effects, in regards to the inflammatory parameters of adjuvant-induced arthritis. Subcutaneous injection of ES into *Zusanli* acupoint significantly reduced ankle inflammation, when compared to saline injection, as demonstrated by reduction in articular index. We previously reported that *Hominis placenta* (HP) herb acupuncture, the water extract of human placenta, showed about 25% recovery of articular indexes of the treated rats, compared to those of non-treated arthritic control rats (13). With respect to the articular index of polyarthritis treatment in rats, it was concluded that ES herb-acupuncture produced two-fold therapeutic efficiency compared to HP herb-acupuncture. Although the arthritic symptoms of adjuvant-induced arthritis rats were apparently alleviated by ES treatment into *Zusanli*, AIA-induced weight loss was not recovered in the ES/Z group. This may be due to the

accelerating effects of ES extracts on body weight loss and metabolic energy consumption (16, 17, 29, 32–34).

In addition, the mRNA expressions of TNF- α , IL-1 β , and IL-6 genes, which were highly stimulated in the knee joints of arthritic rats, were decreased to almost the same levels as those in normal rats, after ES treatment into ST36. Several inflammatory cytokines, particularly TNF- α , IL-1 β , and IL-6, are known to play a key role in mediating the destruction of cartilage and bone in arthritis; and they have thus been investigated as important biological targets to develop drugs for use in the treatment of active rheumatoid arthritis (1, 33, 35). The anti-arthritic effect of ES herb-acupuncture might be related to the interference of these cytokines' pathogenic roles within the joints. The ES injection into non-acupoint did not show any anti-inflammatory effect on adjuvant-induced arthritis, contrary to the injection into Zusanli. It did not reduce the arthritic edema of the joint and the expressions of inflammatory cytokines, which were verified by measuring arthritis index and analyzing the mRNA expressions of TNF- α , IL-1 β , and IL-6, respectively.

It was concluded that the subcutaneous injection of ES extracts exerts a medicinal effect when injected only into the appropriate acupoint, such as *Zusanli* (ST36), which implies that the anti-arthritic effect of ES herb-acupuncture was attributed to not only the pharmacological action by ES injection, but also sustained stimulation of the acupoint by the ES injection. This provides experimental evidence for the merits of herb-acupuncture, having both medicinal benefits of acupuncture and pharmacological therapy at once. In conclusion, this study showed that ES herb-acupuncture was synergically effective in alleviating the arthritic symptoms in adjuvant-induced arthritic rat, in regards to joint appearance and mRNA expression profiles of inflammatory cytokines. However, individual studies of the constituents of ES distillates, with regard to the biochemical and pharmacological aspects must be performed to identify its action mechanisms in arthritis.

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